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Synthesis of activated 3′-amino-3′-deoxy-2-thio-thymidine, a superior substrate for the nonenzymatic copying of nucleic acid templates†

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We present a scalable synthesis of 3′-amino-3′-deoxy-2-thio-thymidine-5′-phosphoro-2-methylimidazolide, an activated monomer that can copy adenosine residues in nucleic acid templates rapidly without a polymerase. The sulfur atom substitution enhances the rate of template copying by 5-fold compared with the 3′-amino-3′-deoxy-T monomer, while the 3′-amino monomers exhibit a 2- to 30-fold enhancement compared with their ribonucleotide counterparts.

Nonenzymatic template-directed replication of nucleic acids has been hypothesized to be the mechanism of information transfer in primitive cells prior to the advent of ribozyme polymerases.[1] Early efforts involving high-energy nucleotide monomers such as 5′-phosphoro-2-methylimidazolides (or 2-MeImpNs) (Fig. 1, top) showed that RNA templates consisting of C residues can be copied by 2-MeImpG in hours to days in the presence of divalent cations (typically Mg^{2+}).[2] However, no enzyme-free process has yet been discovered that enables the rapid and efficient copying of mixed-sequence RNA templates with activated ribonucleotide monomers. This problem has stimulated interest in alternative nucleic acids that might exhibit faster replication chemistry; such polymers are of interest both with respect to the origin of life and in the context of designing artificial life forms based on non-biological chemistry. The most promising non-biological nucleic acids are the phosphoramidate polymers, which are assembled from nucleotides with an amino group on the sugar instead of the less nucleophilic hydroxyl. N3′-P5′-linked phosphoramidate DNA[3,4] (3′-NP-DNA, Fig. 1, bottom) stands out as an attractive alternative genetic model because it adopts a helical geometry that is similar to that of A-form RNA.[5] A-form geometry is the preferred conformation for the nonenzymatic template-directed oligomerization of activated ribonucleotides[6,7] most likely because the A-form double helix of RNA brings the 3′-OH group of the primer in line with the leaving group of the incoming monomer. We have previously shown[8] that activated 3′-amino-2′,3′-dideoxynucleotide monophosphates (3′-NH_2-2-MeImpddNs) (Fig. 1, bottom) rapidly polymerize on short, homopolymeric DNA, RNA, and locked nucleic acid (LNA) templates. We also found[9] that replacing 3′-amino-T with 3′-amino-2-thio-T enhances the rate and fidelity of 3′-NP-DNA synthesis in the copying of DNA, RNA and 3′-NP-DNA templates. However, further progress in the

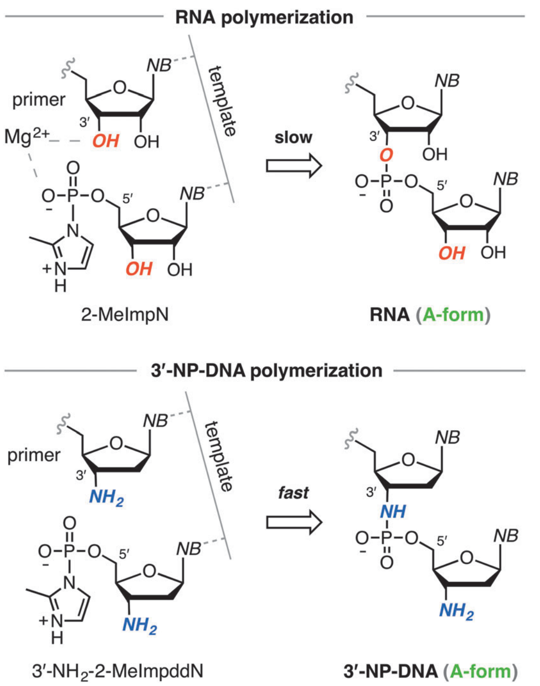

Fig. 1 Template-directed polymerization of RNA (top) and 3-NP-DNA (bottom). *NB* denotes nucleobase.

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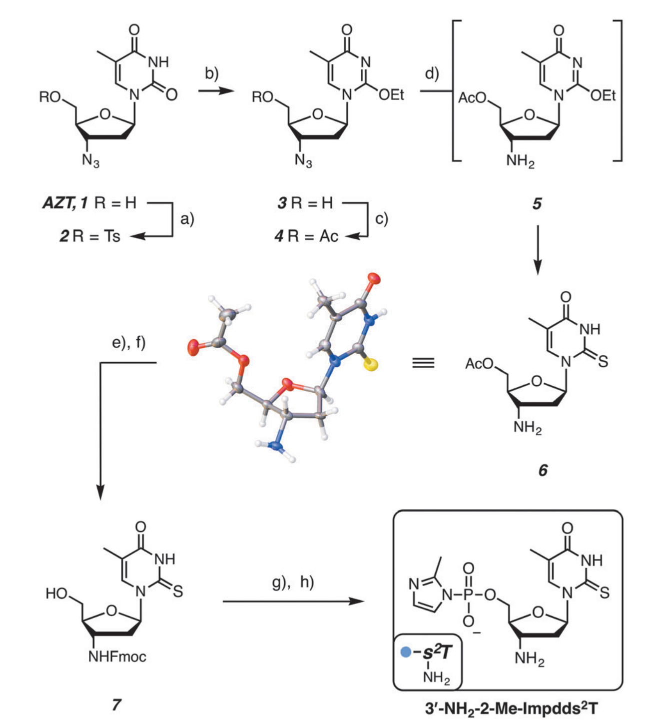
development and quantitative analysis of this system has been hindered by lack of access to the critical substrate, the 5′-phosphoro-imidazolide of 3′-amino-2-thio-T (3′-NH_2-2-MeImp-dds^{2}T), which could previously be synthesized only in small quantities because of the expensive starting material and the very low yield of the desired product.

Here, we present a concise and scalable synthesis of 3′-amino-2-thio-T and the corresponding 5′-phosphoro-imidazolide, 3′-NH_2-2-MeImpdds^{2}T. We also present the first quantitative investigation of template-copying kinetics using this activated nucleotide. Our results show that nonenzymatic 3′-NP-DNA synthesis using the 2-thio modified 3′-amino-T phosphoro-imidazolide monomer is significantly faster than with unmodified 3′-amino-T, and is also considerably faster than RNA synthesis with activated U and 2-thio-U.

Our earlier attempts to establish a concise synthetic route to 3′-NH_2-2-MeImpdds^{2}T involved regioselective thio-carbonylation[10] of 4-*O*-protected 3′-azido-3′-deoxy-thymidine. However, these substrates afforded only deglycosylation products using Lawesson's reagent, an observation later reported[11] for 4-*O*-mesityl-3′,5′-*O*-di(TBS)-thymidine. We then studied nucleophilic ring opening of 2-thio-2,3′-cyclonucleosides by azides at the 3′-position, but observed little to no conversion (by ^{1}H NMR analysis) of the substrates in the presence of either excess LiN_3 or $TMSN_3$ accompanied by various Lewis acids [*e.g.*, $Hg(OAc)_2$, $Er(OTf)_3$ and $Yb(O\text{-}iPr)_3$].



Scheme 1 Synthesis of 3′-NH_2-2-MeImpdds^{2}T. Reaction conditions: (a) TsCl, pyr, 0 to 20 °C, 6 h, 73%; (b) DBU, EtOH, 85 °C, 24 h, 81%; (c) DMAP (cat.), Ac_2O, 20 °C, 12 h, >95%; (d) H_2S (gas), TMG, pyr, 0 to 20 °C, 16 h, 53%; (e) 7N NH_3 in MeOH, 20 °C, 92%; (f) Fmoc-OSu, Na_2CO_3 (aq), pyr, 0 to 20 °C, 6 h, 78%; (g) 1. $POCl_3$, 2,6-lutidine, $PO(OMe)_3$, 3 Å MS, 0 to 20 °C, 2 h; 2. 2-Me-imidazole, 2 h, 30%; (h) piperidine, DMF, 0 °C, 0.5 h, 85%. X-ray structure: H: white, C: gray, O: red, N: blue, and S: yellow.

Our successful strategy to access 3′-NH_2-2-MeImpdds^{2}T (Scheme 1) commenced with the 5′-tosylation[12] of 3′-azido-3′-deoxythymidine (AZT) **1**.[13] Intramolecular displacement of the 5′-tosylate by the C2-oxyanion formed in the presence of a Brønsted base (*e.g.*, DBU) yielded the 2,5′-*O*-anhydro-cyclo-nucleoside, and subsequent ring-opening in refluxing ethanol afforded the 2-ethoxythymidine **3** in 59% overall yield from **1**. After converting **3** into the acetate **4**, we were able to incorporate the sulfur atom into the nucleobase using H_2S in the presence of tetramethylguanidine (TMG)[14] (see Fig. S1 in the ESI† for details of the reaction setup). ^{1}H NMR analysis of an aliquot of the crude reaction mixture after 1 hour revealed that **4** was fully consumed, while two new species were formed: 3′-amino-2-ethoxythymidine **5** and 3′-amino-2-thio-thymidine **6**, in a molar ratio of 2 : 1 (**5**/**6**). The relative abundance of **6** continued to increase as the reaction progressed [up to *ca.* 1 : 3 (**5**/**6**) after 6 hours]. We did not observe (by either ESI or ^{1}H NMR analysis) the formation of any 3′-azido-2-thio-thymidine, suggesting that the incorporation of sulfur was slower than the reduction of the 3′-azide, and that **6** was likely formed from **5**. The structure of the 2-thio nucleoside **6** was confirmed by both ^{1}H–^{1}H gCOSY NMR spectroscopy and X-ray crystallography (see the ESI†). We then converted **6** into the phosphoroimidazolide precursor **7** *via* 5′-deacetylation and Fmoc protection of the 3′-amine. A one-flask 5′-*O*-phosphorylation and 2-methyl-imidazolide synthesis, followed by the removal of Fmoc, provided 3′-NH_2-2-MeImpdds^{2}T in 26% overall yield from **7**.

With 3′-NH_2-2-MeImpdds^{2}T in hand we proceeded to carry out nonenzymatic primer extension experiments to quantitatively interrogate the effect of 2-thio substitution on RNA template-copying rates (Fig. 2). We used a primer/template complex composed of a DNA primer strand ending in a 3′-NH_2-G and a complementary RNA template strand containing a 5′-C_2A_4 overhang. pK_a of the protonated 3′-amine of 3′-amino-2′,3′-dideoxy-2-thio-thymidine is 7.5 (see the ESI†), similar to that reported for 3′-amino-2′,3′-dideoxy-T (7.7).[15] 3′-NH_2-2-MeImpddNs tends to undergo intra-molecular cyclization due to the proximity of the primary 3′-amine group to the phosphorus electrophile.[8] Because the half-life ($t_{1/2}$) of 3′-NH_2-2-MeImpddT is 1.2 h[8] and that of 3′-NH_2-2-MeImpdds^{2}T is 1.3 h (see the ESI† for details) under optimized primer extension conditions [100 mM 1-(2-hydroxyethyl)-imidazole, pH 7.5, 4 °C], we tracked primer extension only up to a maximum of 1 h. We determined observed rate constants k_{obs} for the first step of the primer extension by following the loss of unreacted primer over time (Fig. 2).

At a 10 mM initial concentration of 3′-NH_2-2-MeImpddT (Fig. 2, left), k_{obs} of primer extension was 0.42 h^{-1} (Table 1, entry 1). Notably, 2-thio modification led to about a 5-fold rate enhancement (Fig. 2, right), such that k_{obs} for 10 mM 3′-NH_2-2-MeImpdds^{2}T was 1.92 h^{-1} (Table 1, entry 2). This increase likely results from the additional stabilization induced into the primer/template duplex afforded by the formation of a s^{2}T:A base pair compared to a canonical T:A base pair,[16] as well as the more 3′-endo-like sugar puckering of 2-thio-nucleotide, which is the favoured sugar conformation in nonenzymatic primer extension reactions.[17] The k_{obs} values for reactions containing the activated ribonucleotides[18] 2-MeImpU, 2-MeImps^{2}U and its ribo-T analog 2-MeImps^{2}T were all

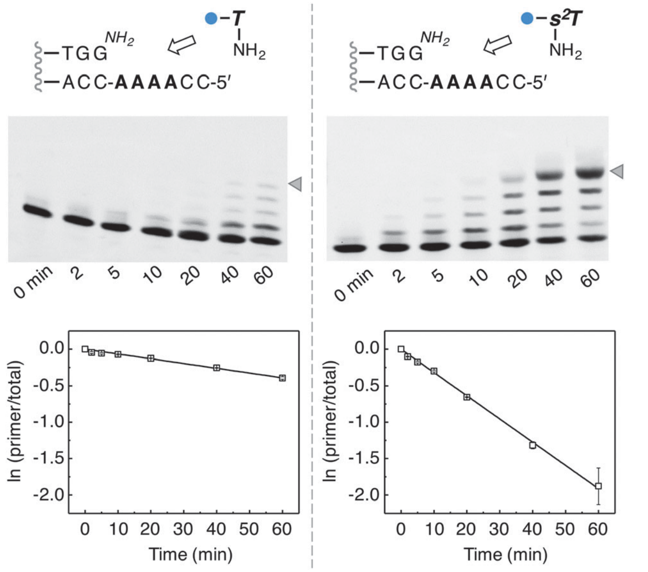


Fig. 2 Kinetics of copying a $r(A)_4(C)_2$ template with 3′-NH_2-2-MeImpddT (left) and 3′-NH_2-2-MeImpdds^{2}T (right), in the presence of 100 mM 1-(2-hydroxyethyl)-imidazole, at pH 7.5 and 4 °C. Reactions were initiated by addition of monomers and monitored by gel electrophoresis. The triangle indicates the primer +4 product. (bottom) Natural log of the fraction of the unreacted primer plotted against incubation time. Errors were based on two experiments. Primer strand (DNA, 0.2 μM): 5′-(FAM)-AGC-GTG-ACT-GAC-TGG-(NH_2)-3′, obtained enzymatically in *ca.* 85% purity based on LC-HRMS (see the ESI†). Primer concentration was corrected for unreactive oligonucleotide impurity. Template strand (RNA, 1 μM): 5′-CCAAAA-CCA-GUC-AGU-CAC-GCU-3′ RNA.

Table 1 Reaction kinetics measured for 10 mM T/U monomers at 4 °C

Entry	Template	Monomer	k_{obs} (h^{-1})	Relative k_{obs}
1[a]	$r(A)_4(C)_2$	3′-NH_2-2-MeImpddT	0.42 (1)	7
2[a]	$r(A)_4(C)_2$	3′-NH_2-2-MeImpdds^{2}T	1.92 (2)	30
3[b]	$r(A)_6$	2-MeImpU	ND	—
4[b]	$r(A)_6$	2-MeImps^{2}U	0.064 (1)	1
5[b]	$r(A)_6$	2-MeImps^{2}T	0.22 (6)	3

[a] In the presence of 100 mM 1-(2-hydroxyethyl)imidazole. [b] Data obtained from ref. 18. Reactions performed with 200 mM $MgCl_2$ at pH 7.0.

lower than the values for the activated 3′-amino nucleotides described above (Table 1), even though these ribonucleotide polymerizations were assayed in the presence of 200 mM Mg^{2+} to optimize the reactivity. The rate enhancement observed for 2-MeImps^{2}T *vs.* 2-MeImps^{2}U suggests that methylation at the 5-position of 2-thiouracil leads to stronger monomer–primer stacking. Additionally, primer extension reactions with 3′-NH_2-2-MeImpdds^{2}T are 10-fold faster than with the corresponding ribonucleotide, 2-MeImps^{2}T, presumably due to the greater nucleophilicity of the 3′-amine. Remarkably, combining the effect of the 3′-amine and the 5-methyl groups results in an *ca.* 30-fold (Table 1, entries 2 *vs.* 4) faster reaction. Further physical and kinetic characterization will be required to distinguish the contributions of enhanced monomer binding *vs.* enhanced monomer reactivity for these observations.

In conclusion, we have developed a scalable synthesis of a 2-thio modified thymidine monomer, 3′-NH_2-2-MeImpdds^{2}T. Our synthetic route provided this highly reactive nucleotide in sufficient amounts to perform quantitative measurements of nonenzymatic RNA template-copying rates for the first time. Our results show that 3′-NH_2-2-MeImpdds^{2}T can polymerize on a DNA/RNA primer/template complex significantly faster than any other U or T monomer that has been reported thus far.

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